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Biomarkers and fluctuating asymmetry as indicators of pollution-induced stress in two hole-nesting passerines

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Summary

1. The stress caused by air pollutants was studied at biochemical, morphological and ecological levels in the Pied Flycatcher, *Ficedula hypoleuca*, and Great Tit, *Parus major*, nestlings in 10 study sites along the pollution gradient of a copper smelter.
2. First, stress was measured using four biomarkers from blood and liver: ethoxyresorufin *O*-deethylase (EROD) enzyme, haemoglobin, stress protein Hsp70 and delta-aminolevulinic acid dehydratase (ALA-d) enzyme. Second, the amount of fluctuating asymmetry (FA) in the length of the 3rd primary and the outermost rectrix (in *P. major*) and the length and thickness of the tarsus were measured. These stress indicators were further examined in relation to the breeding performance of birds, i.e. the ecological response.
3. EROD activity was increased in *F. hypoleuca* nestlings near the pollution source. In *P. major*, it was not related to the pollution gradient but correlated well with the proportion of starved nestlings, i.e. the nutritional stress during the nestling period. The variations in haemoglobin, Hsp70 and ALA-d enzyme activity were not significantly related to pollution gradient.
4. The tarsus length of *F. hypoleuca* nestlings and primary length of *P. major* nestlings showed increased asymmetry in the vicinity of the pollution source. Breeding success decreased towards the pollution source in both species.
5. In *F. hypoleuca*, the pollution-related stress was verified at all three target levels. In *P. major*, pollution-related effects were found at morphological and ecological levels but not at the biochemical level, which suggests that direct toxic effect of heavy metals was not the main mechanism in this species.

Key-words: Breeding success, heavy metals, pollution stress

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Introduction

Air pollutants can pose stress for birds at several functional levels. At its extreme, populations can become extinct from a polluted area. At moderate levels there is usually detectable damage, which is often connected to the most vulnerable part of the life cycle, reproduction (e.g. Matsumura 1975; Scheuhammer 1991; Peakall 1992; Hoffman 1995). From the conservation point of view it would be valuable to be one step ahead and detect the detrimental effects even earlier. Measuring toxic concentrations from birds will help in many cases but may not be enough for two reasons. First, it is often not clear at all what xenobiotics should be measured and the effective levels may not be known. Second, the stress caused by air pollutants may be indirect, e.g. caused by the decreased amount of food, and consequently cannot be evaluated by measuring tissue concentrations of pollutants. In their review article, Brouwer, Murk & Koehman (1990) emphasized the importance of integrated studies of biochemical, physiological and ecological approaches in assessment of pollution impacts.

In recent years, biomarkers have proved to be a good tool in detecting early biological changes caused by environmental pollution. Biomarkers are defined as measurable xenobiotically induced changes in biochemical processes (e.g. enzyme activity) or compounds (Peakall 1992). Another biological early warning sign is the developmental instability during the ontogeny of an individual that is shown by many animals under various environmental stress situations (Leary & Allendorf 1989; Parsons 1990; Pankakoski *et al.* 1994). One indicator of developmental stability is fluctuating asymmetry (FA), which measures deviations from symmetrical development of morphological characters (Palmer & Strobeck 1986).

In this study we measured the pollution-induced stress in the nestlings of two hole-nesting passerines, the Pied Flycatcher, *Ficedula hypoleuca*, and the Great

Tit, *Parus major*, in different parts of the pollution gradient around a point source discharge of heavy metals and sulphuric oxides. Our main objective is to compare the stress responses of these two insectivorous bird species. We present the bone tissue concentrations of one major pollutant, lead, as a measure of direct accumulation of heavy metals. To understand the impact mechanisms, stress was measured at biochemical, morphological and ecological levels. As indicators of physiological stress four biomarkers were used: EROD enzyme, haemoglobin, stress protein Hsp70 and ALA-d enzyme (the last of which is considered specific for lead exposure). Stress effects at the morphological level were studied by measuring fluctuating asymmetry in three/four bilateral morphological characters: primary length, rectrix length (in *P. major*), tarsus length and tarsus width. Breeding success and nestling mortality were used as ecological measures of stress.

Materials and methods

STUDY AREA

The data were collected in the surroundings of the town Harjavalta (61°20′N, 22°10′E), SW Finland (Fig. 1). The main source of local air pollutants is a factory complex producing copper, nickel and fertilizers in the centre of the town. Sulphuric oxides and heavy metals, especially Cu, Zn, Pb and Ni, are common pollutants in the area (Kubin 1990; Jussila & Jormalainen 1991). In 1996, particulate atmospheric release of sulphuric oxides was 3243 tonnes and that of the metals 49, 5, 2 and 1 tonnes, respectively. Sulphur and metal contents in the environment decrease exponentially with increasing distance from the factory complex (Fritze *et al*. 1989; Jussila & Jormalainen 1991; Eeva & Lehikoinen 1996; Jussila 1997). Ten study sites, each with 45–55 nestboxes, were established during 1991–95 along the air pollution gradient in three main directions (SW, SE and NW) away from the copper smelter complex up to a distance of 6 km. A more detailed description of the study area and the pollution gradient has been given in Eeva, Lehikoinen & Pohjalainen (1997).

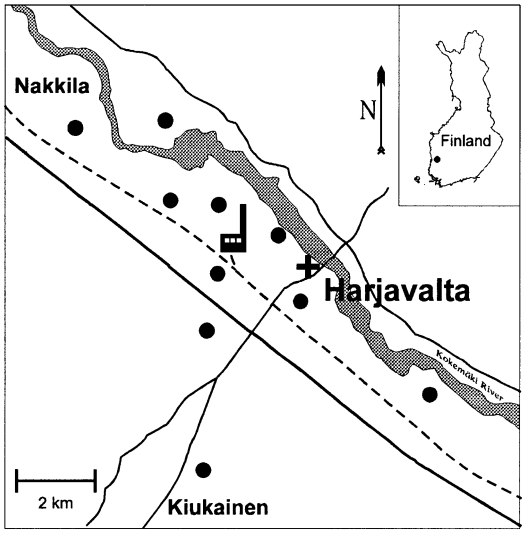

Fig. 1. Location of the study area and 10 study sites (●) around the copper smelter. The shape and extension of the pollution gradient have been described in Eeva *et al*. (1997).

MORPHOMETRIC MEASURES

During the breeding period, nestboxes were visited at least once a week to measure nestlings and to determine laying and hatching dates. Hatching dates were confirmed by extra visits to the nests when necessary. For both species, 33 nests were randomly chosen (two to five nests per study site) for asymmetry and biomarker measurements. Nestlings were weighed at a constant age (11 ± 1 days for *F. hypoleuca* and 15 ± 1 days for *P. major*), measured bilaterally for the length of the 3rd primary and the outermost rectrix (for *P. major* only), and for the length and the width of tarsus. Body mass was measured with a spring balance to the nearest 0·1 g. The feather lengths were measured with a ruler to the nearest 0·5 mm and the length and the width of tarsus were measured with a sliding calliper to the nearest 0·1 mm. All the measurements were made by the same observer to avoid extra variation in the data. In statistical analyses of morphometric parameters, brood means are used as replicates to avoid pseudoreplication.

The morphological characters did not show directional asymmetry since the left-minus-right values did not differ significantly from zero (two-tailed pairwise *t*-tests, $P > 0{\cdot}10$ for all characters in both species). Nor did they show antisymmetry, since the distributions were unimodal and did not significantly deviate from normality (Shapiro–Wilk test: $P > 0{\cdot}05$ for all characters in both species). Because the age of nestlings varied from 10 to 12 days in *F. hypoleuca* and from 14 to 16 days in *P. major* all the morphological measures were standardized to the constant ages of 11 and 15 days, respectively, by linear regressions. This standardization had no effect on the actual difference between left and right character values.

In general, all the morphological characters were positively correlated with each other for both species (the exception was tarsus width, which showed significant correlations only with body mass) but, on the other hand, were not correlated with asymmetry measures. This means that size scaling of asymmetry measures is not needed and, consequently, the unscaled and unsigned left-minus-right value (|L-R|) was used as an index of FA (see Palmer & Strobeck 1986). Since the distributions of unsigned FA-values for primary and rectrix lengths were skewed, a square root transformation was performed for these variables before the analyses to achieve equal variances.

TISSUE SAMPLES

After the morphological measurements one nestling of intermediate size was chosen from each brood for

the biomarker study. By selecting an intermediate-sized nestling we wanted to avoid measuring stress from very small individuals, which owing to sibling competition may be in a stressed position all the time. On the other hand, the largest nestling may rarely be exposed to nutritional stress. The permission to collect nestlings was obtained from the local authorities. Nestlings were decapitated on-site and a blood sample for haemoglobin measurement was taken from the jugular vein with a 25-µl microcapillar tube. Capillary tubes were put into 4 ml of haemoglobin reagent and shaken strongly. Haemoglobin was determined using the cyanmethaemoglobin method. The carcass was dissected immediately after blood sampling and the liver was stored in plastic tubes into a liquid nitrogen container for enzyme analyses. The rest of the carcass was frozen and preserved for heavy metal analyses. The removal of nestlings was not taken into account when we calculated the values for nestling mortality (dead nestlings × 100/hatchlings) and breeding success (fledglings × 100/clutch-size). This did not, however, bias the data because the effect was the same for each brood.

Since, in many cases, blood (erythrocyte) ALA-d activity is a more sensitive indicator of lead exposure than liver ALA-d activity, a separate set of blood samples for ALA-d assay was collected from 10 11-day-old *F. hypoleuca* and 10 15-day-old *P. major* nestlings from the vicinity of the factory (< 2 km) and from distant sites (> 5 km). These samples were taken by puncturing the brachial vein and allowing the blood to flow into a heparinized 25-µl microcapillar tube.

MIXED FUNCTION OXIDASES

Mixed function oxidases (MFO) are major components in the defence of organisms against toxic chemicals (Peakall 1992). MFOs convert organic xenobiotics into a more hydrophilic form, facilitating excretion in bile. Activity of mixed function oxidases is affected by a wide variety of toxic compounds. The activity of ethoxyresorufin O-deethylase (EROD) enzyme was measured fluorimetrically from hepatic microsomes according to the method described by Burke & Mayer (1974), with minor modifications. Liver samples were rinsed with ice-cold 1·15% KCl and homogenized in 0·25-M sucrose with a motor-driven Potter Elvehjelm glass–Teflon homogenizer (Research Products International, Mount Prospect, IL, USA). The liver homogenate was centrifuged at 8000 g for 20 min, and the resulting supernatant was further centrifuged at 100 000 g for 1 h. The microsomal pellet was resuspended in SET buffer: 0·25 M sucrose, 5·4 mM EDTA (ethylenedinitrilo tetra-acetic acid), 66 mM Tris, pH 7·4. Samples were stored at –70 °C until assayed. The reaction mixture contained 1930 µl Tris-HCl buffer (pH 7·4), 2 µl ethoxyresoreufin and 30 µl microsomal sample. Reaction was started by adding 40 µl of NADPH (β-nicotinamide adenine dinucleotide phosphate). Samples were excited at 510 nm and the emission was measured at 586 nm at 42 °C with a Perkin Elmer Luminescence Spectrometer LS 50 (Perkin Elmer Ltd, Beaconsfield, UK). Internal standardization was performed by adding resorufin at 50 µM. EROD activity was expressed as nanomoles per minute per milligram of microsomal protein (nmol min^{-1} mg^{-1} protein). Protein concentration was determined by the method of Bradford (1976) using the BioRad Protein Assay Kit (BioRad Laboratories, Hercules, CA, USA). One exceptionally high value (1·82 nmol min^{-1} mg^{-1} protein) for *P. major* was omitted as an outlier from further analysis.

HEAT SHOCK PROTEINS

Stress proteins (heat shock proteins, Hsps) help cells in recovering from stress situations by correcting misconfigurations in protein structures. Heat shock proteins have been used as biomarkers because their synthesis is induced by various toxicants, including heavy metals (Morimoto, Tissiéres & Georgopoulos 1990; Delmas *et al*. 1996). The induction of Hsp70 was measured using the Western blot analysis. Samples were prepared by homogenizing hepatic tissue at 4 °C in 150 mM Tris-HCl, pH 7·8/0·5 mM phenylmethylsulfonylfluoride. Samples were then centrifugated at 13 000 ***g*** for 30 min at 4 °C and the pellet was discarded. Samples were stored at –70 °C until used. Protein concentration was determined using Bradford's (1976) method. Equal amounts of hepatic proteins (15 µl) were loaded on 8% SDS (sodium dodecyl sulfate)–polyacrylamide gels and separated electrophoretically according to Laemmli (1970). The separated proteins were blotted (Bio-Rad Semi-Dry Electrophoretic Transfer Cell, Richmond, CA, USA) onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The blots were treated with a monoclonal mouse anti-Hsp70 antibody, 3a3 (Affinity Bioreagents, Golden, CO, USA). This antibody recognizes both the constitutive and the stress-inducible forms of Hsp70-protein. The presence of the primary antibody was detected by incubating the membrane in horseradish peroxidase-conjugated sheep antimouse Ig (Amersham, UK). The antibody–antigen complexes were visualized by the enhanced chemiluminescence reaction (ECL, Amersham).

HEAVY METAL ANALYSES

Metallic lead is one of the main pollutants in the study area and it is known to be toxic for birds especially during their early development (Pain 1995). Because the symptoms in growing birds in the previous study (Eeva & Lehikoinen 1996) were consistent with lead poisoning, lead concentrations from the femur bones of nestlings were measured. Bones were dried overnight at 60 °C, weighed and dissolved into a 1 : 2 mixture of H_2SO_4 and HNO_3. Lead concentrations were analysed with an atomic absorption spectrophotometer using the graphite furnace technique. As a standard, commercial bovine liver liquid series containing 4·0, 12·0 and 24·0 µg lead l^{-1} was used.

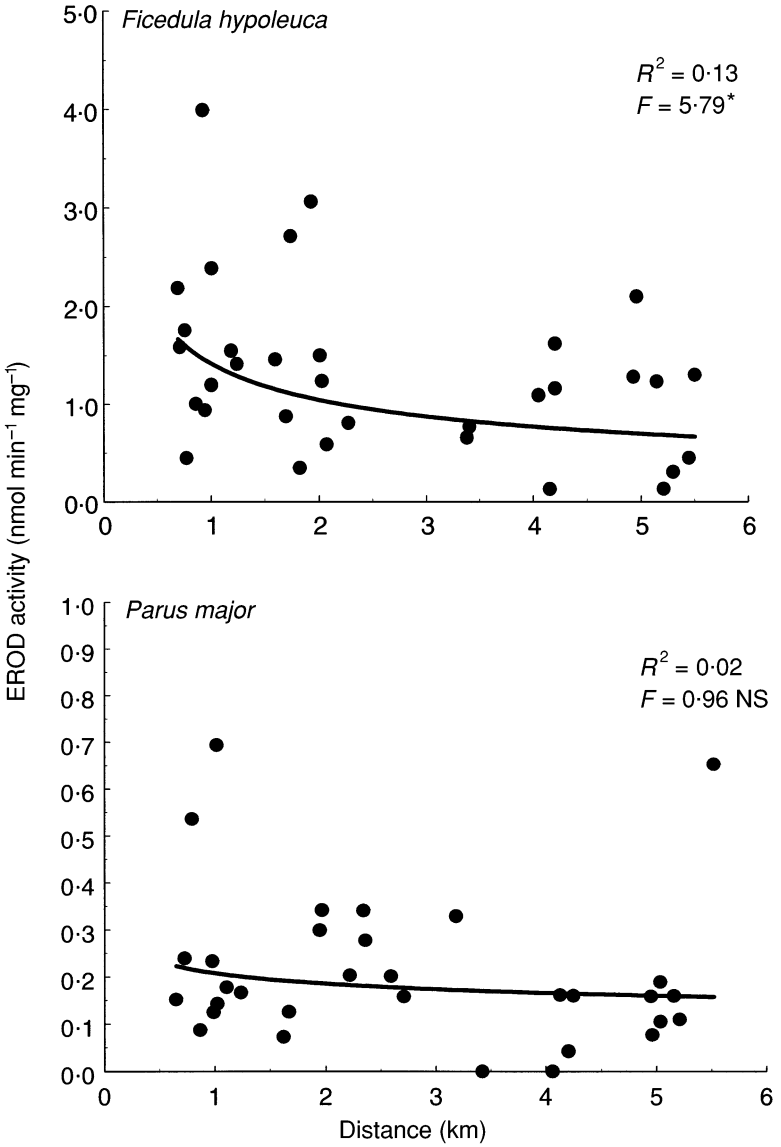

Fig. 2. The EROD enzyme activity (nmol min^{-1} mg protein^{-1}) in *Ficedula hypoleuca* and *Parus major* nestling livers at different distances from the pollution source. The second order power functions ($y = a \times x^{-b+c\times\ln x}$) and their F-values: $^*P < 0{\cdot}05$; NS = not significant. Note the different scales on y-axes.

ALA-D DETERMINATIONS

Delta-aminolevulinic acid dehydratase (ALA-d) is an enzyme in the haem biosynthetic pathway, and is extremely sensitive to inhibition by lead (Scheuhammer 1989). ALA-d is not readily inhibited by other heavy metals (Peakall 1992) and can thus be used as a specific biomarker for lead exposure. Hepatic and blood ALA-d activity was measured by the method described by Scheuhammer (1987a). The hepatic tissue was homogenized in ice-cold 0·9% NaCl and centrifuged at 8000 ***g*** for 15 min at 4 °C to remove cell debris. Blood samples (25 µl) were mixed with four volumes of 1% saponine/0·1% Triton X-100, shaken vigorously and stored in liquid nitrogen. For activity determinations, a 50-µl sample was added to a reaction mixture containing 0·25 M MES (morpholinoethanesulfonic acid) buffer, 25 µl deionized water and 15 mM 5-aminolevulinic acid hydrochloride. Samples were then incubated at 42 °C for 1 h, and the reaction was stopped by adding 100 µl 0·4 M trichloroacetic acid/60 mM Hg^{2+} ($HgCl_2$). After that samples were centrifuged at 15 000 ***g*** for 5 min, and modified Erlich's reagent was added to the supernatant at (1 : 7·5). After 10 min, the absorbance was read at 555 nm with a Perkin Elmer Lambda Bio UV/Vis Spectrometer. Activity was expressed as picomoles per hour per milligram of protein. Activity calculations were made assuming that the molar extinction of the porphobilinogen–pyrrole complex is 6×10^4 (Mauzerall & Granick 1965). Protein concentration was determined by the method of Bradford (1976). One blood sample of *P. major* was discarded from further analysis owing to exceptionally high protein content. ALA-d values were log-transformed before the analyses to normalize distributions.

STATISTICS

Between-species differences and the dependence of response variables on the pollution gradient were tested with the GLM procedure (with SS3) of the SAS statistical system (SAS Institute Inc. 1989). Distance to the pollution source was used as the independent variable in most analyses because we were interested in not only direct toxic stress effects, but also indirect effects that cannot be directly quantified by measuring pollutant concentrations in birds' tissues. The normality of residuals was checked for each model using the UNIVARIATE procedure of SAS. In correlation tests, the Pearson correlation coefficient was used, unless otherwise stated. Predated or otherwise destroyed nests were omitted from all analyses. All means are given with their standard errors (± SE).

Results

The mean EROD enzyme activity (nmol min^{-1} mg^{-1} protein) in the liver was 1·31 (± 0·15, $n = 33$) in *F. hypoleuca* nestlings and 0·21 (±0·029, $n = 32$) in *P. major* nestlings. The difference between the two species was highly significant (Kruskal–Wallis test; $\chi^2 = 37{\cdot}4$, $P < 0{\cdot}0001$). The dependence of EROD activity on the degree of pollution was tested with regression models using distance to the pollution source and nestling mortality (dead nestlings × 100/hatchlings) as independent variables. To distinguish between pollution stress and 'natural' nutritional stress (due to starving) nestling mortality was included in the model as an indicator of overall stress during the nestling period. The distance to the pollution source explained significant variation in EROD activity in *F. hypoleuca* ($F_{2,28} = 5{\cdot}25$, $P = 0{\cdot}030$) but not in *P. major* ($F_{2,27} = 0{\cdot}03$, $P = 0{\cdot}87$). In *F. hypoleuca* the highest enzyme activity values were found in the vicinity of the pollution source, but the variation was relatively high along the whole gradient (Fig. 2). In *P. major*, mortality was higher in those nests where EROD

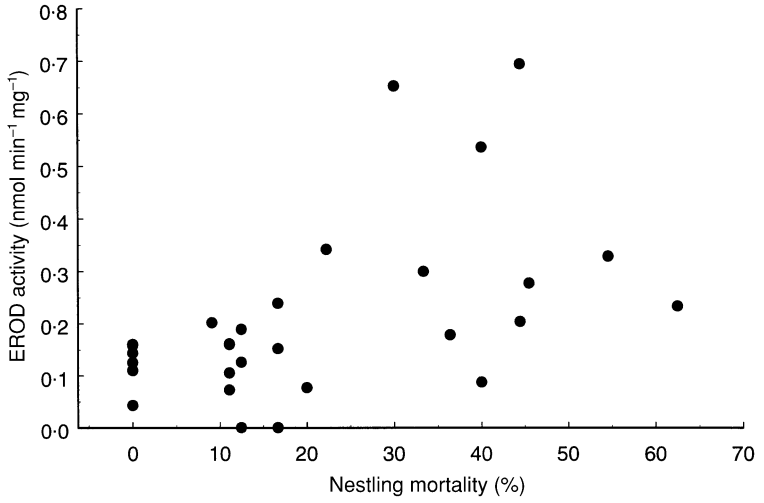

Fig. 3. The relationship between *P. major* EROD enzyme activity (nmol min^{-1} mg $protein^{-1}$) and percentage nestling mortality (dead nestlings × 100/hatchlings). $N = 30$.

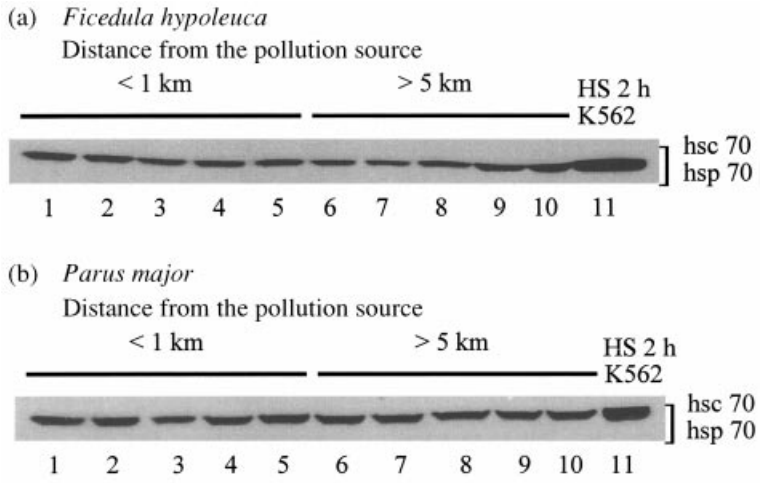

Fig. 4. Levels of hsp70-protein in *Ficedula hypoleuca* (a) and *Parus major* (b) hepatic samples. Results from five nearest (< 1 km from the pollution source; lanes 1–5) and five most distant (> 5 km from the pollution source; lanes 6–10) sampling sites are shown. Heat shock treated human cell line K562 cells were used as a positive control (lane 11).

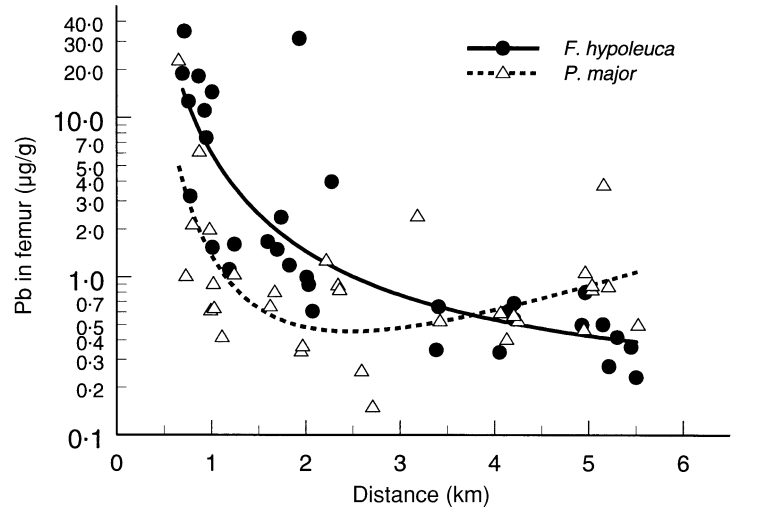

Fig. 5. Lead concentrations (µg/g, dry mass) in the femurs of *Ficedula hypoleuca* ($n = 33$) and *Parus major* ($n = 32$) nestlings at different distances from the pollution source. The second order power functions ($y = a \times x^{-b+c\times\ln x}$): *F. hypoleuca*, $R^2 = 0{\cdot}44$, $F = 34{\cdot}2$, $P < 0{\cdot}0001$; *P. major*, $R^2 = 0{\cdot}61$, $F = 9{\cdot}86$, $P < 0{\cdot}001$.

activity was also high ($F_{2,27} = 8{\cdot}84$, $P = 0{\cdot}0061$, Fig. 3), whereas this relationship was not found in *F. hypoleuca* ($F_{2,28} = 0{\cdot}39$, $P = 0{\cdot}54$, data not shown).

The mean blood haemoglobin concentration was 104·8 g l^{-1} (±7·3, $n = 25$) for *F. hypoleuca* and 105·7 g l^{-1} (±6·0, $n = 32$) for *P. major*, and the means did not differ between the species (ANOVA, $F_{1,55} = 0{\cdot}01$, $P = 0{\cdot}92$). Haemoglobin concentration did not depend on the distance from the pollution source in either species (regression, *F. hypoleuca*: $F_{2,22} = 0{\cdot}73$, $P = 0{\cdot}40$; *P. major*: $F_{2,27} = 0{\cdot}72$, $P = 0{\cdot}40$), neither was it related to nestling mortality in *F. hypoleuca* ($F_{2,22} = 0{\cdot}78$, $P = 0{\cdot}39$). Instead, there was a marginally significant trend of haemoglobin values being higher in those *P. major* broods where nestling deaths occurred more frequently ($F_{2,27} = 3{\cdot}98$, $P = 0{\cdot}056$).

The mean ALA-d enzyme activity in liver was 239·2 pmol h^{-1} mg^{-1} (±63·8, $n = 33$) for *F. hypoleuca* and 381·2 pmol h^{-1} mg^{-1} (±64·1, $n = 32$) for *P. major*, the latter value being significantly higher (ANOVA, $F_{1,63} = 8{\cdot}98$, $P = 0{\cdot}0039$). Surprisingly, hepatic ALA-d activity did not depend on the femur lead concentrations in either species (regression, *F. hypoleuca*: $F_{1,29} = 1{\cdot}46$, $P = 0{\cdot}24$; *P. major*: $F_{1,28} = 0{\cdot}28$, $P = 0{\cdot}27$). Consequently, ALA-d activity was not dependent on the distance from the pollution source (regression, *F. hypoleuca*: $F_{2,28} = 1{\cdot}24$, $P = 0{\cdot}27$; *P. major*: $F_{2,27} = 0{\cdot}44$, $P = 0{\cdot}51$), or nestling mortality in a brood (*F. hypoleuca*: $F_{2,28} = 0{\cdot}05$, $P = 0{\cdot}82$; *P. major*: $F_{2,27} = 0{\cdot}18$, $P = 0{\cdot}68$). The mean ALA-d activity in blood was 227·2 pmol h^{-1} mg^{-1} (±19·6, $n = 10$) for *F. hypoleuca* and 395·3 pmol h^{-1} mg^{-1} (±28·4, $n = 9$) for *P. major*, and was not different between the sites close to the factory and distant sites (ANOVA, *F. hypoleuca*: $F_{1,8} = 1{\cdot}74$, $P = 0{\cdot}22$; *P. major*: $F_{1,7} = 0{\cdot}01$, $P = 0{\cdot}93$). EROD activity, haemoglobin and hepatic ALA-d activity were not correlated with each other in either species (Spearman correlation, $P > 0{\cdot}05$ in all tests).

The lack of effect was also obvious for Hsp70 protein in both bird species. There was a high constitutive level of Hsp70, but no induction was observed in any of the samples examined (Fig. 4, lanes 1–10). In the positive control sample (heat shock treated human erytroleukaemia cell line K562 cells) both constitutively expressed (Hsc70) and inducible forms (Hsp70) of Hsp70 were detected (Fig. 4, lane 11).

The concentration of lead in femur increased towards the pollution source in both bird species (Fig. 5). The lead concentration was higher in *F. hypoleuca* than in *P. major* close (< 3 km) to the pollution source (ANOVA: $F_{1,37} = 13{\cdot}6$, $P = 0{\cdot}0007$) whereas in distant sites (> 3 km) the opposite was true (ANOVA: $F_{1,24} = 7{\cdot}28$, $P = 0{\cdot}013$).

Breeding success (fledglings × 100/clutch-size) was positively correlated with distance from the pollution source in both species (Fig. 6).

Among the morphological characters (body mass, primary and rectrix length, tarsus length and width) of nestlings only the body mass of *P. major* and tarsus

Table 1. The means (± standard error, SE) of brood mean left and right character values (mm) in *P. major* ($n = 48$ broods) and *F. hypoleuca* ($n = 38$ broods) nestlings. Linear regression tests for the dependence of absolute asymmetry (unsigned left–right value) on the distance to the pollution source

	Parus major					*Ficedula hypoleuca*				
	Left		Right		Distance†	Left		Right		Distance†
	$\bar{x}$	SE	$\bar{x}$	SE	H_0: $\beta = 0$	$\bar{x}$	SE	$\bar{x}$	SE	H_0: $\beta = 0$
Primary length	29·93	0·568	30·10	0·569	*	23·34	0·435	23·37	0·441	NS
Rectrix length	19·29	0·626	19·48	0·631	NS	–	–	–	–	
Tarsus length	19·33	0·103	19·31	0·097	NS	17·36	0·081	17·31	0·077	*
Tarsus width	1·47	0·007	1·46	0·007	NS	1·16	0·007	1·15	0·008	NS

†A square root transformation was made for the asymmetry values of primaries and rectrices to normalize distributions before the regression tests.

*$P < 0·05$; NS = not significant. Brood means were used as replicates. A sequential Bonferroni correction over the number of tests.

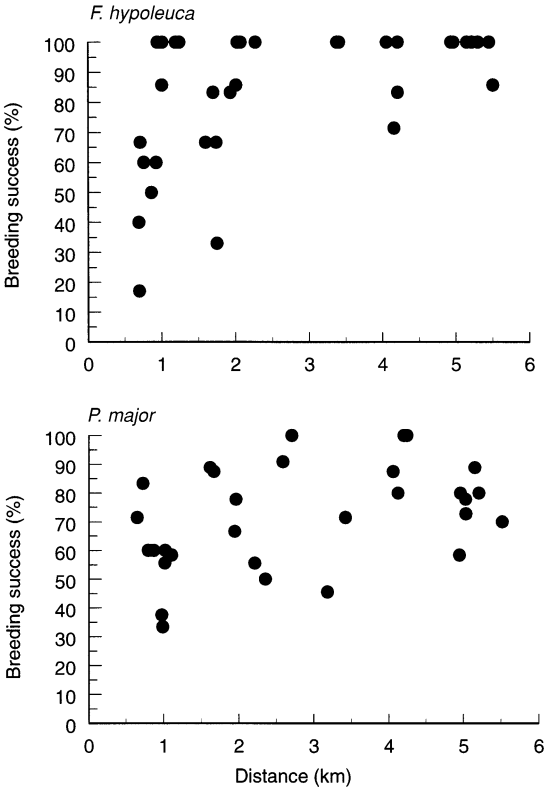

Fig. 6. The breeding success (fledglings × 100/clutch size) of *Ficedula hypoleuca* ($n = 33$) and *Parus major* ($n = 30$) at different distances from the pollution source. (Spearman correlation; *F. hypoleuca*: $n = 33$, $r_s = 0·59$, $P = 0·0003$; *P. major*: $n = 30$, $r_s = 0·38$, $P = 0·040$). Destroyed nests were omitted.

width of *F. hypoleuca* were correlated with the distance to the pollution source (a sequential Bonferroni correction over the number of correlation tests: df = 47, $r = 0·49$, $P = 0·011$; df = 37, $r = 0·46$, $P = 0·009$, respectively). *Parus major* nestlings were thus heavier and *F. hypoleuca* nestlings had thicker tarsa far from the pollution source than close to it.

The amount of fluctuating asymmetry along the pollution gradient was studied with linear regressions (Table 1). The length of the 3rd primary of *P. major* nestlings and the tarsus length of *F. hypoleuca* nestlings showed higher asymmetry closer to the pollution source than farther away (Fig. 7). Except for the marginally significant correlation between FA in tarsus and primary feathers of *F. hypoleuca* nestlings, asymmetry measures were not correlated with each other in either species. Therefore, the relationship between breeding success and the amount of fluctuating asymmetry was tested by including all three/four asymmetry measures as explanatory factors in multiple regression models. In both species, the asymmetry in tarsus length was related to breeding success (*P. major*: $F_{1,45} = 12·37$, $P = 0·0011$; *F. hypoleuca*: $F_{1,37} = 5·03$, $P = 0·0316$). Interestingly, this relationship was positive for *P. major* and negative for *F. hypoleuca*. The negative relationship in *F. hypoleuca* was because of their greater asymmetry and poor breeding success in the vicinity of the pollution source: when sites closer than 2 km from the smelter were omitted from the analysis this relationship disappeared. The positive correlation in *P. major* is understandable if there is a trade-off between the amount of FA and the number of nestlings. This seems to be the case in *P. major*, because in this species the number of nestlings at time of measurement correlated strongly with the amount of FA ($r = 0·41$, $P = 0·0042$). Instead, in *F. hypoleuca*, this correlation was not found ($r = -0·23$, $P = 0·16$).

Discussion

Three very different measures (biochemical, morphological and ecological) showed that *F. hypoleuca* nestlings suffered stronger stress during their development in the vicinity of the copper smelter than farther away. First, bone tissue lead concentrations and liver EROD activity were increased near the smelter. Second, tarsus length showed increased asymmetry in the polluted area. Third, breeding success was lowered near the smelter. These effects, however, were restricted to a relatively small area (about 1–2 km) around the pollution source. The result is consistent with earlier observations from the same area, which showed that the breeding success of *F. hypoleuca* was very poor in the vicinity of the factory, where the

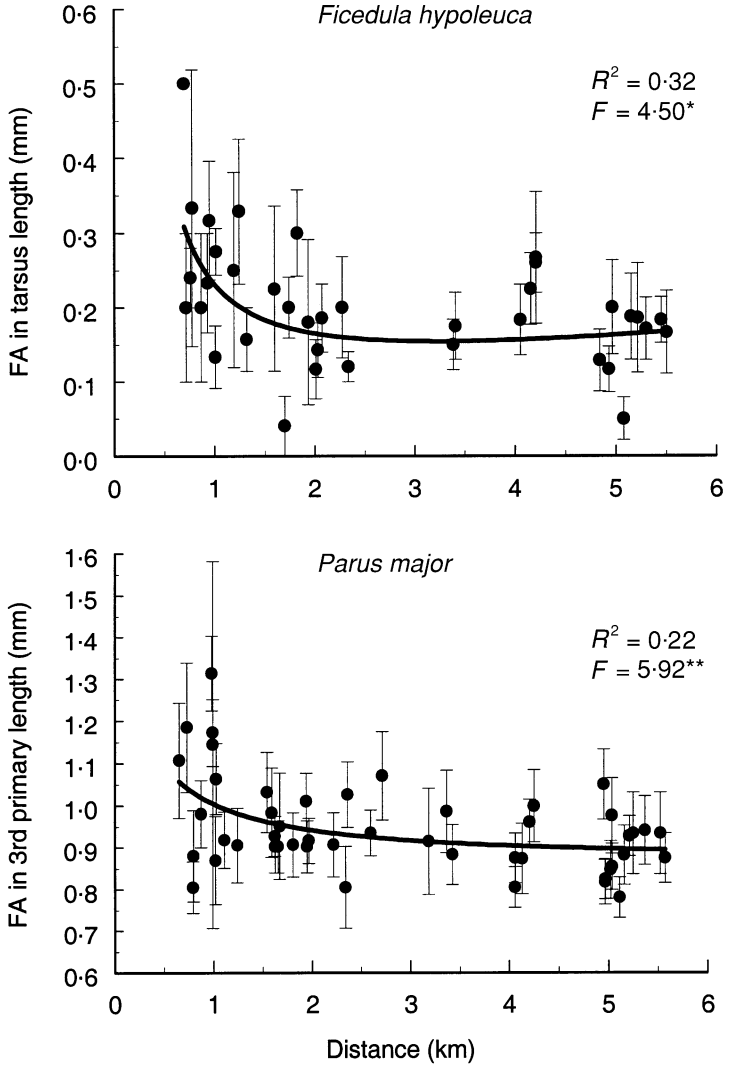

Fig. 7. Fluctuating asymmetry (FA) in tarsus length of *Ficedula hypoleuca* nestlings and 3rd primary length of *Parus major* nestlings at different distances from the pollution source. The second order power functions ($y = a \times x^{-b+c \times \ln x}$) and their F-values: *$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

amount of heavy metals in nestling diet was high (Eeva & Lehikoinen 1996). That an increased amount of FA in tarsus length but not in feathers was observed was not surprising given that defectively developed legs and defective egg shells were known to occur in this area (Eeva & Lehikoinen 1995, 1996). Both abnormalities, as well as the decreasing thickness of tarsus observed in this study, are related to calcium metabolism, which is known to be detrimentally affected by heavy metals (e.g. Scheuhammer 1987b).

Parus major nestlings showed lower bone tissue lead concentrations than *F. hypoleuca* nestlings in the polluted area. Furthermore, *P. major* nestlings did not show increased EROD enzyme activity, which suggests that xenobiotics do not pose a similar problem for this species as for *F. hypoleuca*. The difference between species can be related to different food choice but also physiological differences may play a role. However, one sign of increased stress was also found in *P. major* nestlings: FA in the length of 3rd primary increased close to the pollution source. In another study from the same area it was found that *P. major* nestlings suffered from the secondary effect of air pollution, lowered availability of insect food (Eeva, Lehikoinen & Pohjalainen 1997). The same trend was also observed in the present study since the body mass of nestlings decreased towards the pollution source. Food deprivation has been experimentally demonstrated to increase FA in the primary feathers of the European Starling, *Sturnus vulgaris* L. (Swaddle & Witter 1994; but see Björklund 1996). It is thus possible that increased FA in primaries of *P. major* nestlings close to the copper smelter is caused by secondary effects of air pollution via reduced insect food.

Increased hepatic EROD activity was found in *F. hypoleuca* in the vicinity of the copper smelter. However, we are not aware of any study that has demonstrated an effect of heavy metals on EROD activity. Instead, the MFO system is well known to be affected by a wide range of hydrocarbons, via their effect on the aryl hydrocarbon receptor (Peakall 1992). It is, however, possible that combustion-derived hydrocarbons are emitted from the smelter in sufficient quantities to cause the observed effect on enzyme activity. For example, in 1995 the smelter used 16 000 tonnes of oil and 4900 tonnes of coal in the enrichment process. Concentrations of some hydrocarbons (chlorophenols, chlorobenzenes, PCBs) were measured in exhaust fumes in 1991 but the Finnish Environment Institute considered the emitted amounts insignificant at that time. Unfortunately there are no data available on other hydrocarbon [e.g. polycyclic aromatic hydrocarbons (PAHs), dioxins] emissions or concentrations in birds. The correlation between EROD activity and nestling mortality in *P. major*, however, suggests that EROD activity may also vary owing to factors not necessarily related to hydrocarbon pollution.

According to Scheuhammer (1987b) Pb levels over 5 μg/g (dry mass) in bones of adult wild birds would be indicative of some degree of increased environmental exposure, and Pain (1995) summarized that in most species of wild vertebrates from uncontaminated areas, bone Pb concentrations are usually less than 20 μg/g (dry mass). In growing nestlings these values are obviously smaller and detrimental effects are also manifested at lower concentrations than in adult birds. On the basis of observed bone deformities in nestling legs, the highest Pb (10–40 μg/g, dry mass) concentrations observed in the present study were toxic for growing nestlings. Similar concentrations were found to cause breeding failures in *F. hypoleuca* in a study of Nyholm (1994). Here it is important to remember that the composition of diet strongly affects the toxicity of lead (Six & Goyer 1970; Scheuhammer 1996). Dietary calcium is known to be one of the most important factors influencing lead absorption (Pain 1995), and acidifying emissions (mainly SO_2) have decreased the number of calcium rich food items (e.g. snail shells) in the vicinity of the pollution source (Eeva & Lehikoinen 1996). In a previous

study additional calcium carbonate was given to some *F. hypoleuca* broods living in a polluted area (Eeva 1996). Experimental enhancement of Ca in food reduced the occurrence of bone deformities from 75% (control broods) to zero (treatment).

ALA-d activity was not suppressed in either study species, indicating that the lead exposure from the smelter did not affect the enzyme activity. Interestingly, the disturbances in calcium metabolism and bone development appear in the present case to be a more sensitive indicator of heavy metal exposure than either the induction of Hsp70 or blood or liver ALA-d activity. This is somewhat surprising since ALA-d activity is considered to be a very sensitive indicator of lead poisoning. It is, however, possible that ALA-d is not a good biomarker for lead exposure of nestlings, in which the haematological system is under development and ALA-d activity is generally high owing to intense biosynthesis of haem (see Grue, O'Shea & Hoffman 1984; Grue *et al*. 1986; Peakall 1992). This seems to be the case for *F. hypoleuca* and *P. major* too. Our preliminary measurements of unexposed nestlings and adults showed that ALA-d activity was 3·3 times higher in *F. hypoleuca* nestlings and 1·8 times higher in *P. major* nestlings than in adult birds. Inherently high ALA-d activity of nestlings may thus make it difficult to detect any suppression induced by lead, especially when dietary concentrations are moderate.

On the basis of the observed effects we consider it likely that *F. hypoleuca* suffers from the food rich in heavy metals near the pollution source, and thus shows increased FA in bones (femur) because of impaired calcium metabolism. The *P. major* nestlings seem to be more stressed by food limitation and the detrimental effects manifest in decreased body mass and increased FA in primaries. By using biomarkers together with ecological data on breeding our earlier assumption on different impact mechanisms in the two bird species (Eeva, Lehikoinen & Pohjalainen 1997) could be confirmed, but more information on the expression of different kinds of xenobiotics and other stress factors is clearly needed to use biomarkers or FA efficiently in detecting early pollution impacts.

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